REMARKS/ARGUMENT

Claim 1 has been amended to recite that the permeate solution comprises an organic fluid that is substantially immiscible with water, incorporating the subject matter of claims 3 and 4. Claims 3 and 4 have been cancelled, and the dependencies of the remaining claims have been adjusted. Accordingly, no new matter is introduced in making these amendments.

Claims 1, 5, 8-9, and 11-13 remain in the application.

Claims 1, 3-5, 9, 12, and 13 stand rejected under 35 U.S.C. 102(b) as being anticipated by Zhu et al. (Zhu). This rejection is respectfully traversed for the following reasons.

First, claim 1 requires, *inter alia*, that the method use a membrane made from a hydrophobic material having a hydrophilic feed side and a permeate side, wherein the permeate side of the membrane has a water contact angle of greater than 90° and the feed side has a water contact angle of less than 70°. Zhu does not disclose such a membrane.

Zhu discloses a hydrophilic PVDF, low protein binding membrane (paragraph 2.2). Such membranes are hydrophilic on both the feed side and the permeate side of the membrane. Water readily wets the surface of both sides of such membranes, having a water contact angle of less than 70°. The Examiner contends, referring to paragraph 2.2 at page 400, that "the receptor-side of this membrane is coated with lecithin in dodecane—which makes the receptor (or permeate-side) hydrophobic." But the pertinent portion of paragraph 2.2 at page 400 actually reads as follows:

> *In an artificial membrane permeability experiment, a 96-well filtration plate (hydrophilic PVDF, low-protein binding, Millipore, Bedford, MA) was used for the artificial membrane support and as the receiver plate. The filter material in each well of the filtration plate was wetted with 5 μL of the artificial membrane solution, which consisted of 1% egg lecithin in n-dodecane. Then the filtration plate was securely placed on top of a donor plate (Dynex, Middlesex, UK) which was pre-filled with donor solutions (100-200 μM drug solution in phosphate buffer, pH 5.5 or 7.4) in each well. Equal volumes of blank receiving solution were quickly added to the wells of the filtration plate.*

Thus, Zhu discloses that "the filter material" in each well was "wetted" with the lecithin in dodecane solution. This does not suggest or imply that only the receptor-side of the membrane is coated with the hydrophobic "artificial membrane solution." Indeed, one skilled in the art would realize that wetting a hydrophilic PVDF filter material with this solution would result in a hydrophobic surface on both the feed-side and the permeate-side of the membrane—not just the permeate side, as asserted by the Examiner.

Claim 1 as amended also requires that the permeate solution comprise an organic fluid that is substantially immiscible with water. Zhu does not disclose or suggest this. Referring again to Zhu paragraph 2.2 above, the donor solution (i.e., the feed solution) is drug in a phosphate buffer at pH 5.5 or 7.4. Zhu states that "volumes of blank solution" are then added to the wells of the filtration plate (that is, to the receiving or permeate solution). One skilled in the art would understand that a "blank" solution is the same aqueous phosphate buffer solution as used in the feed, but containing no drug. Thus, the permeate solution is an aqueous solution, and not an organic fluid, as required by claim 1.

The Examiner contends that the permeate solution in Zhu is octanol, referring to the introduction on page 399 and to paragraph 2.4 on page 401. However, these

sections refer to octanol-water partition coefficients and to octanol-buffer distribution coefficients, respectively. They do NOT refer to the permeate solution.

Because Zhu does not disclose a microporous membrane made from a hydrophobic material that has (*i*) a hydrophilic feed side and (*ii*) a permeate solution comprising an organic fluid substantially immiscible with water, both as recited in claim 1, there can be no anticipation of claim 1 by Zhu. Since all the remaining claims ultimately depend from claim 1, they are likewise not anticipated by Zhu.

Claims 1, 3-5, 8, 9, and 11-13 stand rejected under 35 U.S.C. 103(a) as being obvious over Zhu in combination with Kallury US 7,468,281 (Kallury). Kallury discloses the use of hollow-fiber membranes for the purification, concentration, and analysis of samples (abstract). However, neither Zhu nor Kallury disclose, teach, or suggest the use of a microporous membrane made from a hydrophobic material that has a hydrophilic feed side, nor the use of a permeate solution comprising an organic fluid that is substantially immiscible with water. Because of this, amended claim 1 would not have been obvious over Zhu in combination with Kallury. The remaining claims depend from amended claim 1, and are likewise not obvious over Zhu in combination with Kallury.

The following remarks pertain to the Examiner's comments in the Advisory Action of July 31, 2009.

The Examiner states:

> *Regarding the Zhu reference, the egg lecithin was applied to the inside of the well, which means the membrane at the bottom of the well on the outside is hydrophilic. In addition, the egg lecithin membrane is a phospholipid membrane, which means it itself is having one side hydrophilic and other hydrophobic (page 400).*

In response, it should be noted that Zhu actually states the following at page 400:

> *the filter material in each well of the filtration plate was wetted with 5 μL of the artificial membrane solution, which consisted of 1% egg lecithin in n-dodecane (emphasis added).*

Thus, Zhu discloses that the artificial membrane solution is used to wet the filter material. One of ordinary skill in the art would understand that this means the pores of the filter material were filled with the artificial membrane solution, and that the filter material—both the top and the bottom, would have the properties of the liquid in the pores. That being the case, the wetted filter material would have the same contact angle on the top as on the bottom, which does not satisfy the limitations of claim 1, which calls for a contact angle of greater than about 90° on the permeate side and less than about 70° on the feed side.

The Examiner further contends that the egg lecithin referred to by Zhu is "a phospholipid membrane." But Zhu states nothing of the sort, instead stating the egg lecithin/dodecane solution was used to wet the artificial membrane. Even accepting the Examiner's contention that the egg lecithin/dodecane solution constitutes a phospholipid membrane for purposes of argument, the Examiner's position is undermined by the known properties of phospholipids. Specifically, reference is made to the enclosed excerpt from Stryer, *Biochemistry*, pages 228-291 (3rd Ed 1988). As noted at pages 288-289, the favored structure for most phospholipids is a bimolecular sheet or lipid bilayer, as depicted in Figure 12-10. Note that Figure 12-10 depicts such a membrane as having a middle section of hydrocarbon (or hydrophobic) tails sandwiched between two outer layers of polar (or hydrophilic) groups. In other words, a phospholipid membrane does not have one side that is hydrophilic and one side that is hydrophobic, as maintained by the Examiner.

In the Advisory Action the Examiner further states:

Regarding the blank receiving solution, there is no indication in the reference that it is only the phosphate buffer; the Examiner submits that it only means the receiving solution does not con tain the drug.

Thus, the Examiner concedes that Zhu is silent regarding the composition of the receiving (or permeate) solution. However, some insight into the types of receiving solutions analyzed by Zhu can be gained from page 400 of Zhu where HPLC analysis of the receiving solutions is described. Here, Zhu states that a YMC basic HPLC column is used with a gradient of mobile phases consisting of (a) water and 0.1% formic acid; and (b) 95% acetonitrile, 5% water and 0.1% formic acid. Mobile phases of water and acetonitrile are typically used in HPLC analysis for determining the concentration of a solute in an aqueous sample. See Meyer, *Practical High-Performance Liquid Chromatography*, pages 144-155 (2d Ed 1997) enclosed herewith; the Examiner's attention is specifically directed to page 146 and page 153, Fig. 10.6. Given this, one of ordinary skill would understand that the "blank" receiving solution was an aqueous buffer solution.

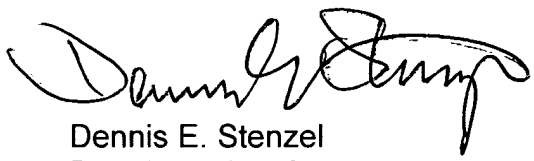
Finally, the Examiner states:

Regarding the Kellury [sic] reference, even if the well is made of hollow fibers, it is still a multi-well plate; and applicant's claims do not discriminate the hollow fibers [sic]. Kellury [sic] teaches that making the membrane surface hydrophilic or hydrophobic will depend on the analyte.

In response, applicants reiterate that the secondary reference Kallury does not disclose or suggest the use of a microporous membrane made from a hydrophobic material that has a hydrophilic feed side, nor the use of a permeate solution comprising an organic fluid that is substantially immiscible with water, as called for in claim 1. Thus, claim 1 is not rendered obvious by the combination of Kallury and Zhu.

Early and favorable reconsideration is respectfully requested.

Respectfully submitted,



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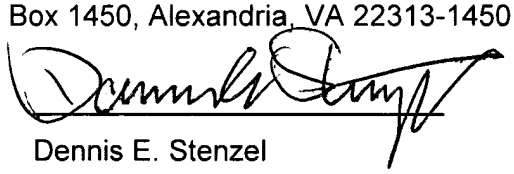

Dennis E. Stenzel

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PHOSPHOLIPIDS AND GLYCOLIPIDS READILY FORM BILAYERS

The repertoire of membrane lipids is extensive, perhaps even bewildering at first sight. However, they possess a critical common structural theme: *membrane lipids are amphipathic molecules*. They contain both a *hydrophilic* and a *hydrophobic* moiety (Table 12-1).

Table 12-1
Hydrophobic and hydrophilic units of membrane lipids

Membrane lipid	*Hydrophobic unit*	*Hydrophilic unit*
Phosphoglycerides	Fatty acid chains	Phosphorylated alcohol
Sphingomyelin	Fatty acid chain and hydrocarbon chain of sphingosine	Phosphoryl choline
Glycolipid	Fatty acid chain and hydrocarbon chain of sphingosine	One or more sugar residue
Cholesterol	Entire molecule except for OH group	OH group at C-3

Let us look at a space-filling model of a phosphoglyceride, such a phosphatidyl choline (Figure 12-6). Its overall shape is roughly rectangular. The two fatty acid chains are approximately parallel to one another, whereas the phosphoryl choline moiety points in the opposite direction. Sphingomyelin has a similar conformation (Figure 12-7). The sugar group of a glycolipid occupies nearly the same position as the phosphoryl choline unit of sphingomyelin. Therefore, the following shorthand has been adopted to represent these membrane lipids. The hydrophilic unit, also called the *polar head group*, is represented by a circle, whereas the hydrocarbon tails are depicted by straight or wavy lines (Figure 12-8).

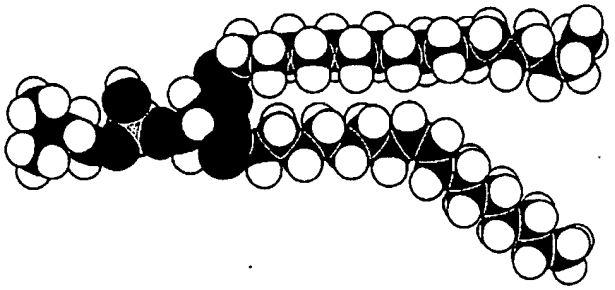


Figure 12-6
Space-filling model of a phosphatidyl choline molecule.

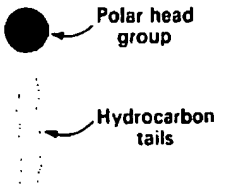
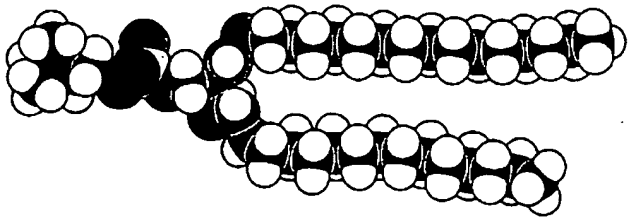


Figure 12-8
Symbol for a phospholipid glycolipid molecule.

Figure 12-7
Space-filling model of a sphingomyelin molecule.



Now let us consider the arrangement of phospholipids and glycolipids in an aqueous medium. It is evident that their polar head groups will have affinity for water, whereas their hydrocarbon tails will avoid water. These preferences could be satisfied by formation of a *micelle*, a globular structure in which polar head groups are on the surface and hydrocarbon tails are sequestered inside (Figure 12-9). Another arrangement that fulfills both the hydrophilic and hydrophobic preferences of these membrane lipids is a *bimolecular sheet*, also called a *lipid bilayer* (Figure 12-10).

The favored structure for most phospholipids and glycolipids in aqueous media is a bimolecular sheet rather than a micelle. The reason is that their two fatty acyl chains are too bulky to fit into the interior of a micelle. In contrast, salts of fatty acids (such as sodium palmitate, a constituent of soap), which contain only one fatty acyl chain, readily form micelles. The formation of bilayers instead of micelles by phospholipids is of critical biological importance. A micelle is a limited structure, usually less than 200 Å in diameter. In contrast, a bimolecular sheet can have macroscopic dimensions, such as a millimeter (10^7 Å). Phospholipids and glycolipids are key membrane constituents because they readily form extensive bimolecular sheets. Furthermore, these sheets serve as permeability barriers, yet they are quite fluid.

The formation of lipid bilayers is a *self-assembly process*. In other words, the structure of a bimolecular sheet is inherent in the structure of the constituent lipid molecules, specifically in their amphipathic

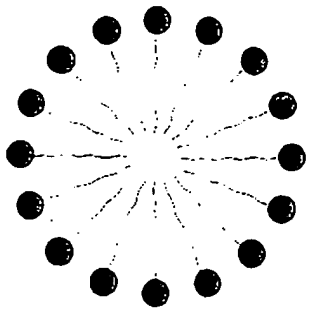


Figure 12-9
Diagram of a section of a micelle formed from ionized fatty acid molecules. Most phospholipids do not form micelles.

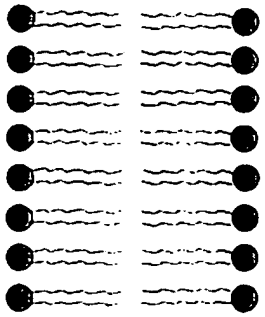


Figure 12-10
Diagram of a section of a bilayer membrane formed from phospholipid molecules.

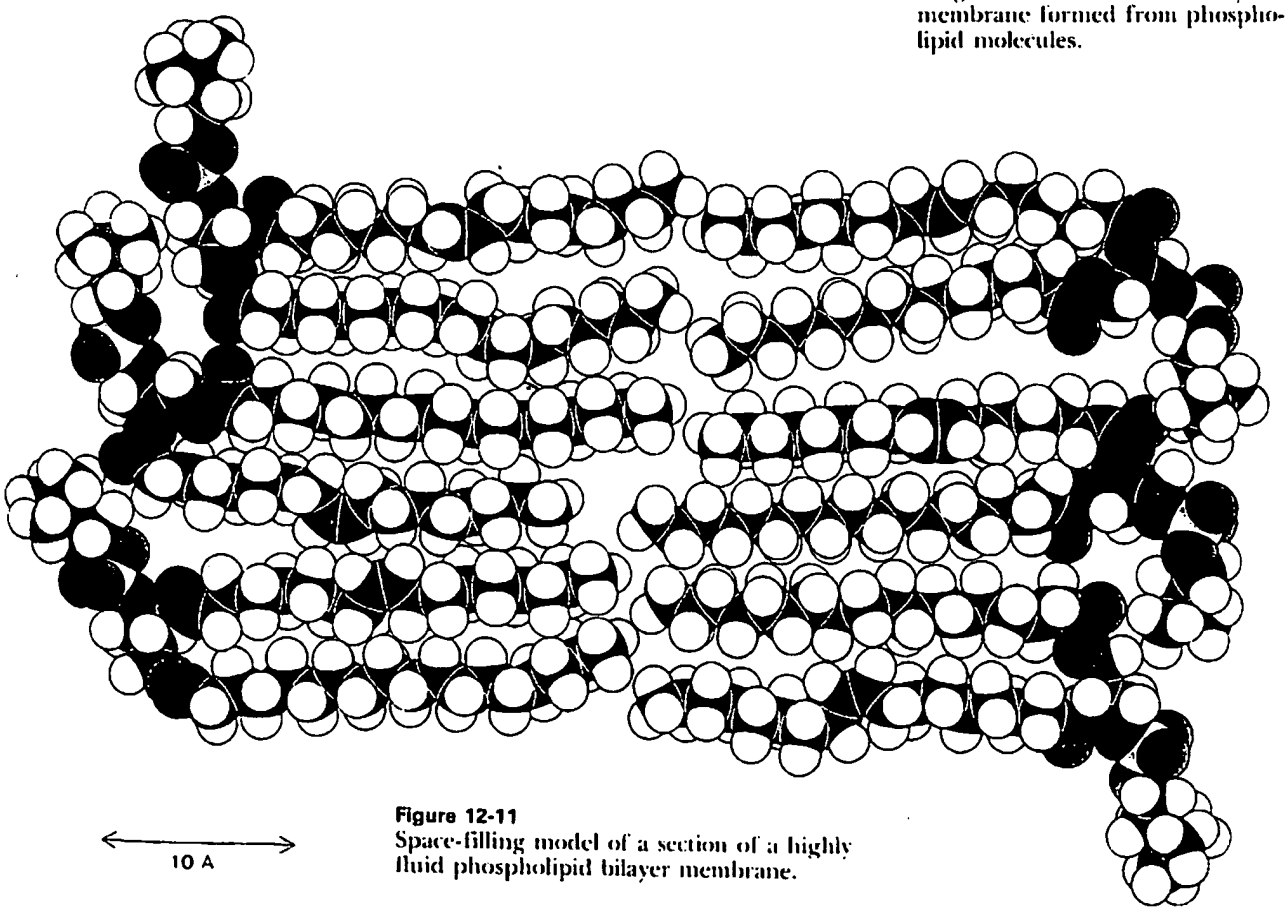


Figure 12-11
Space-filling model of a section of a highly fluid phospholipid bilayer membrane.

character. The formation of lipid bilayers from glycolipids and phospholipids is a rapid and spontaneous process in water. *Hydrophobic interactions are the major driving force for the formation of lipid bilayers.* Recall that hydrophobic interactions also play a dominant role in the folding of proteins in aqueous solution. Water molecules are released from the hydrocarbon tails of membrane lipids as these tails become sequestered in the nonpolar interior of the bilayer. Furthermore, there are *van der Waals attractive forces* between the hydrocarbon tails. These forces favor close packing of the tails. Finally, there are *electrostatic and hydrogen-bonding attractions between the polar head groups and water molecules.* Thus, lipid bilayers are stabilized by the full array of forces that mediate molecular interactions in biological systems.

LIPID BILAYERS ARE NONCOVALENT, COOPERATIVE STRUCTURES

Another important feature of lipid bilayers is that they are *cooperative structures.* They are held together by many *reinforcing, noncovalent interactions.* Phospholipids and glycolipids cluster together in water to minimize the number of exposed hydrocarbon chains. A pertinent analogy is the huddling together of sheep in the cold to minimize the area of exposed body surface. Clustering is also favored by the van der Waals attractive forces between adjacent hydrocarbon chains. These energetic factors have three significant biological consequences: (1) lipid bilayers have an inherent tendency to be *extensive*; (2) lipid bilayers will tend to *close on themselves* so that there are no edges with exposed hydrocarbon chains, which results in the formation of a compartment; and (3) lipid bilayers are *self-sealing* because a hole in a bilayer is energetically unfavorable.

LIPID BILAYERS ARE HIGHLY IMPERMEABLE TO IONS AND MOST POLAR MOLECULES

The permeability of lipid bilayers has been measured in two well-defined synthetic systems: lipid vesicles and planar bilayer membranes. These model systems have been sources of insight into a major function of biological membranes—namely, their role as permeability barriers. The key finding is that lipid bilayers are inherently impermeable to ions and most polar molecules.

Lipid vesicles (also known as *liposomes*) are aqueous compartments enclosed by a lipid bilayer (Figure 12-12). They can be formed by suspending a suitable lipid, such as phosphatidyl choline, in an aqueous medium. This mixture is then *sonicated* (i.e., agitated by high-frequency sound waves) to give a dispersion of closed vesicles that are quite uniform in size. Alternatively, vesicles can be prepared by rapidly mixing a solution of lipid in ethanol with water. This can be accomplished by injecting the lipid through a fine needle into an aqueous solution. Vesicles formed by these methods are nearly spherical in shape and have a diameter of about 500 Å. Larger vesicles (of the order of 10^4 Å, or 1 μm, in diameter) can be prepared by slowly evaporating the organic solvent from a suspension of phospholipid in a mixed solvent system.

Ions or molecules can be trapped in the aqueous compartment of lipid vesicles by forming the vesicles in the presence of these substances

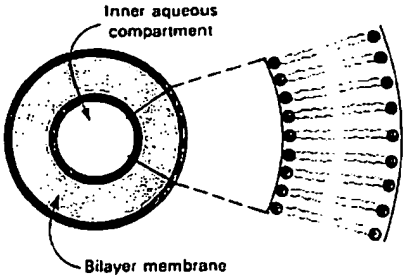


Figure 12-12
Diagram of a lipid vesicle.

(Figure 12-13). For example, if vesicles 500 Å in diameter are formed in a 0.1 M glycine solution, about two thousand molecules of glycine will be trapped in each inner aqueous compartment. These glycine-containing vesicles can be separated from the surrounding solution of glycine by dialysis or by gel-filtration chromatography. The permeability of the bilayer membrane to glycine can then be determined by measuring the rate of efflux of glycine from the inner compartment of the vesicle to the ambient solution. These lipid vesicles are valuable not only for permeability studies. They fuse with the plasma membrane of many kinds of cells and can thus be used to introduce a wide variety of impermeable substances into cells. The selective fusion of lipid vesicles with particular kinds of cells is a promising means of controlling the delivery of drugs to target cells.

Another well-defined synthetic membrane is a *planar bilayer membrane*. This structure can be formed across a 1-mm hole in a partition between two aqueous compartments. Such a membrane is very well suited for electrical studies because of its large size and simple geometry. Paul Mueller and Donald Rudin showed that a large bilayer membrane can be readily formed in the following way. A fine paint brush is dipped into a membrane-forming solution, such as phosphatidyl choline in decane. The tip of the brush is then stroked across a hole (1 mm in diameter) in a partition between two aqueous media. The lipid film across the hole thins spontaneously; the excess lipid forms a torus at the edge of the hole. A planar bilayer membrane consisting primarily of phosphatidyl choline is formed within a few minutes. The electrical conduction properties of this macroscopic bilayer membrane are readily studied by inserting electrodes into each aqueous compartment (Figure 12-14). For example, its permeability to ions is determined by measuring the current across the membrane as a function of the applied voltage.

Permeability studies of lipid vesicles and electrical conductance measurements of planar bilayers have shown that *lipid bilayer membranes have a very low permeability for ions and most polar molecules*. Water is a conspicuous exception to this generalization; it readily traverses such membranes. The range of measured permeability coefficients is very wide (Figure 12-15). For example, Na^+ and K^+ traverse these membranes 10^9 times more slowly than does H_2O. Tryptophan, a zwitterion at pH 7, crosses the membrane 10^3 times more slowly than indole, a structur-

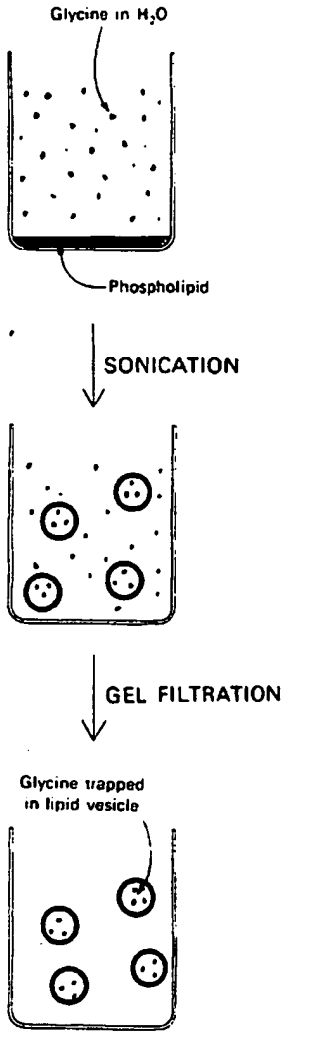


Figure 12-13
Preparation of a suspension of lipid vesicles containing glycine molecules.

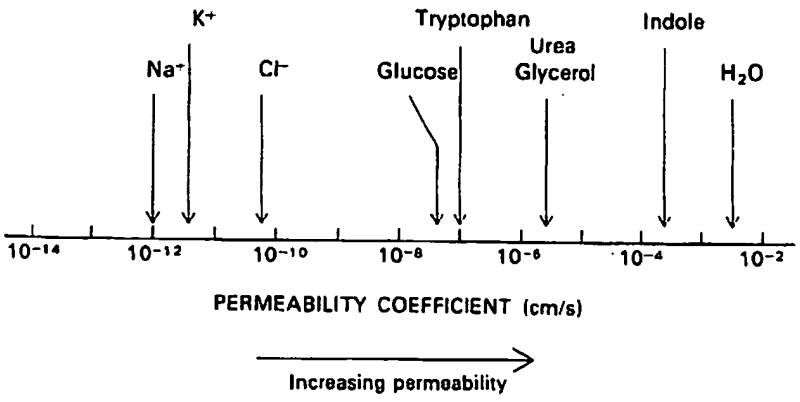


Figure 12-15
Permeability coefficients of some ions and molecules in lipid bilayer membranes.

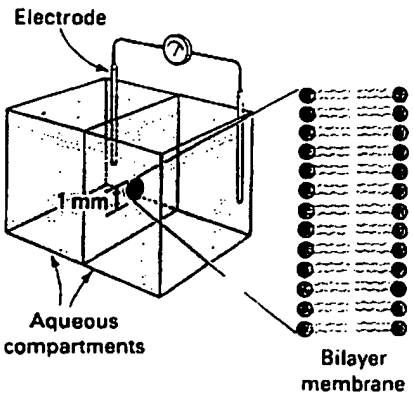


Figure 12-14
Experimental arrangement for the study of planar bilayer membranes. A bilayer membrane is formed across a 1-mm hole in a septum that separates two aqueous compartments.

Practical High-Performance Liquid Chromatography

SECOND EDITION

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University of Berne

JOHN WILEY & SONS
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For Otto

Alles ist einfacher, als man denken kann,
zugleich verschränkter, als zu begreifen ist.

Goethe, *Maximen*

Everything is simpler than can be imagined,
yet more intricate than can be comprehended.

10 Reversed-Phase Chromatography

Reversed-phase chromatography can be classified as a special type of either adsorption or partition chromatography.[1] As this discussion is restricted to chromatography with bonded C_{18} and C_8 brush packings, strictly the subject should be incorporated into Chapter 12, which describes chemically bonded phases. However, we have preferred to treat it as a separate case in view of its extreme importance and widespread use.

10.1 PRINCIPLE

Reversed-phase chromatography is the term used to describe the state in which the stationary phase is less polar than the mobile phase. Chemically bonded octadecylsilane (ODS), an n-alkane with 18 carbon atoms, is the most frequently used stationary phase. C_8 and shorter alkyl chains and also cyclohexyl and phenyl groups provide other alternatives. Phenyl groups are more polar than alkyl groups.

Water is often described as the strongest elution medium for chromatography, but in fact this is only true for adsorption processes. Water may interact strongly with the active centres in silica and alumina, so that adsorption of sample molecules becomes highly restricted and they are rapidly eluted as a result. Exactly the opposite applies in reversed-phase systems: water cannot wet the non-polar (hydrophobic = water-repellent) alkyl groups and does not interact with them in any way. Hence it is the weakest mobile phase of all and gives the slowest sample elution rate. The greater the amount of water in the eluent, the longer is the retention time.

[1]The separation mechanism is not yet really clear. Detailed review on the proposed models: J. G. Dorsey and K. A. Dill, *Chem. Rev.*, 89, 331 (1989).

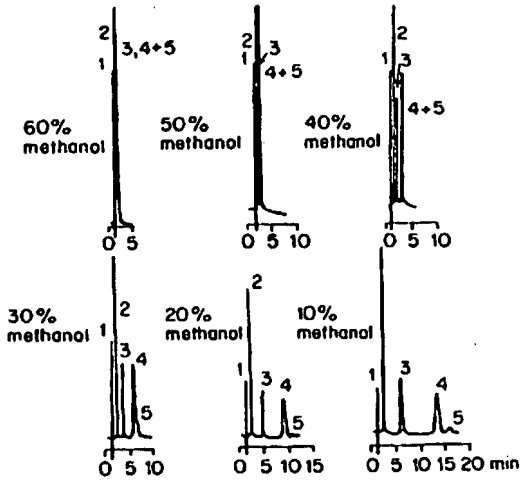

Fig. 10.1 Effect of eluent composition in reversed-phase chromatography (N. A. Parris, *Instrumental Liquid Chromatography*, p. 157).

The chromatogram depicted in Fig. 10.1 demonstrates this point. Benzene (2), chlorobenzene (3), *o*-dichlorobenzene (4) and iodobenzene (5) were eluted on a reversed-phase column by various methanol-water mixtures (1 being the refractive index peak of the solvent used for sample dissolution).

Sample compounds are better retained by the reversed-phase surface the less water soluble (i.e. the more non-polar) they are. The retention decreases in the following order:

aliphatics > induced dipoles (e.g. CCl_4) > permanent dipoles (e.g. $CHCl_3$) > weak Lewis bases[2] (ethers, aldehydes, ketones) > strong Lewis bases (amines) > weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids).

Also, the retention time increases as the number of carbon atoms increases, as shown in Fig. 10.2 for the separation of dec-1-ene (1), undec-1-ene (2), dodec-1-ene (3), tridec-1-ene (4) and tetradec-1-ene (5) on an ODS column. As a general rule the retention increases with increasing contact area between sample molecule and stationary phase, i.e. with increasing number of water molecules which are released during the 'adsorption' of a compound.

Branched-chain compounds are eluted more rapidly than their corresponding normal isomers.

[2]Lewis base = electron donor; Lewis acid = electron acceptor.

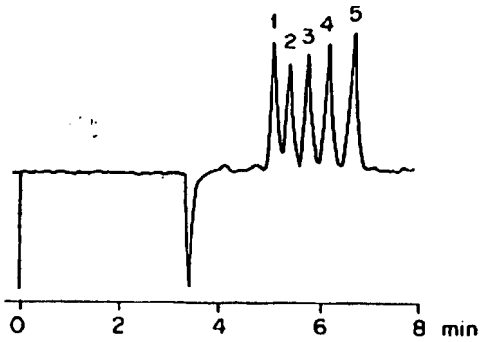

Fig. 10.2 Reversed-phase separation of alkene homologues (Du Pont). Conditions: stationary phase, Zorbax ODS; mobile phase, 0.75 ml min^{-1} tetrahydrofuran–acetonitrile (10:90) (this is an example of non-aqueous reversed-phase chromatography!); IR detector, 3.4 μm.

10.2 MOBILE PHASES IN REVERSED-PHASE CHROMATOGRAPHY

The mobile phase generally consists of mixtures of water or aqueous buffer solutions with various water-miscible solvents, e.g.

methanol	
acetonitrile (toxic!)	
ethanol	decreasing polarity
isopropanol	
dimethylformamide	increasing elution power
propan-1-ol	
dioxane	
tetrahydrofurane	

However, non-aqueous eluents are increasing in popularity for the reversed-phase chromatography of non-polar substances.

As gradients are often involved in reversed-phase chromatography, the solvents used must be extremely pure (see Fig. 19.5). A chromatographic test for the purity of both water and organic eluents has been described,[3] consisting of a well-defined rising and falling gradients which can be used to establish the purity levels of mobile phase components.

The mixtures of water with organic solvents often have a markedly higher *viscosity* than the pure compounds. The situation with the frequently used compositions of methanol–water, tetrahydrofuran–water and acetonitrile–water

[3] D. W. Bristol, *J. Chromatogr.*, **188**, 193 (1980).

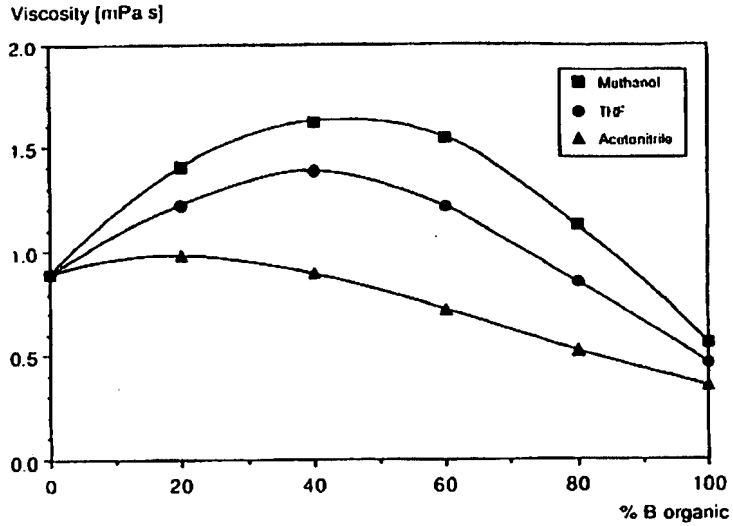

Fig. 10.3 Viscosity of mixtures of water and organic solvents at 25 °C. (Numeric values after J. W. Dolan and L. R. Snyder, *Troubleshooting LC Systems*, Humana Press, Clifton, 1989, p. 85.)

is presented in Fig. 10.3. The viscosity maximum for methanol is obtained with 40% B in water and reaches 1.62 mPa s (25 °C), which is almost the threefold value of methanol or the double value of water. The pressure drop over the column is proportional to the viscosity; therefore it is not constant during a gradient run. High-viscosity maxima are also found with 80% acetic acid in water (2.7 mPa s at 20 °C) and 40% ethanol in water (2.8 mPa s at 20 °C).

Water for HPLC[4] is, unfortunately, not free as it must be specially purified. Ion-exchange water is generally not of sufficient purity and double distillation stills may even increase the content of organics. Water is most effectively purified through a coarse-grained reversed-phase column which is periodically regenerated with acetonitrile; the column is operated as an independent low-pressure unit or, alternatively, it can be positioned on-line between the water storage vessel and the chromatographic system. A photooxidation or multiple-stage water purification system may also be considered or water produced especially for HPLC can be purchased. Sterile water is obtained by filtration through a 0.2 μm filter.

[4] M. Gurkin and J. Ripphahn, *Int. Lab.*, **10**(4), 63 (1980); E. J. Sampson and P. H. Culbreth, *Clin. Chem.*, **28**, 1393 (1982); J. B. Reust and V. R. Meyer, *Analyst (London)*, **107**, 673 (1982); V. V. Berry, *J. Chromatogr.*, **236**, 279 (1982); C. G. B. Frischkorn and H. Schlimper, *Fresenius Z. Anal. Chem.*, **312**, 541 (1982); R. Gabler, R. Hegde and D. Hughes, *J. Liq. Chromatogr.*, **6**, 2565 (1983); V. V. Berry and R. E. Shansky, *J. Liq. Chromatogr.*, **7**, 943 (1984).

Methanol[5] has the disadvantage of producing a relatively highly viscous mixture with water (see above), giving rise to much higher pressures than other mobile phases. If methanol-water mixtures are prepared manually (without a gradient mixer) then both components must be weighed or each one volumetrically determined separately. The considerable volume contraction that occurs produces a solution with a methanol content in excess of 50% by volume from an initial 500 ml of water topped up to 1000 ml with methanol.

Acetonitrile[6] is very expensive, the highly pure 'far-UV' quality being especially so, and it is also toxic (free hydrogen cyanide may even be incorporated). Viscosity properties cause no problems. Note the azeotrope formed with water as a result of regeneration by distillation: it boils at 76.7 °C and contains 84% acetonitrile. Dilute aqueous mixtures can be detoxified by adding sodium hydroxide and hydrogen peroxide. The following reaction takes place on heating:

$$CH_3C{\equiv}N \rightarrow CH_3\underset{\underset{O}{\|}}{C}NH_2 \rightarrow CH_3\underset{\underset{O}{\|}}{C}OH$$

and continues until the amide or the acid is formed, depending on the reaction time and temperature. The solution can then be poured away.

Aqueous sample solutions containing ionized compounds are often used in reversed-phase chromatography. Ionic samples are best separated by ion-exchange or ion-pair chromatography, but the reversed-phase method can be used if the sample mixture contains only weak acids or only weak bases in addition to neutral components. In the former instance, the mobile phase is acidified (see separation of sorbic and benzoic acid in Section 10.3) and in the latter it is made alkaline. The species in question are then in non-dissociated form and do not give rise to chemical tailing. The chromatogram depicted in Fig. 2.15 clarifies this point further.

If this type of ion suppression is not possible for any reason, then a competing compound may be added to the mobile phase.[7] Moreover, ammonium acetate seems to be a 'universal additive' for reversed-phase chromatography.[8]

Anyone used to optimizing k' values will find it relatively easy to construct a nomogram by means of which the change in mobile phase required to achieve the desired k' value can be seen immediately.[9] If higher demands are made it is necessary to use computer-assisted optimization.[10]

[5]N. E. Springarn, C. T. Garvie-Gould and L. L. Vuolo, *Anal. Chem.*, **53**, 565 (1981).
[6]S. M. McCown *et al.*, *Int. Lab.*, **14**(5), 76 (1984); *J. Chromatogr.*, **352**, 465, 483 and 493 (1986).
[7]For addition of aliphatic amines for separation of anilines, see R. A. Hill, *J. Chromatogr.*, **316**, 227 (1984); for separation of tricyclic amines, see J. S. Kiel, S. L. Morgan and R. K. Abramson, *J. Chromatogr.*, **320**, 313 (1985).
[8]C. K. Lim and T. J. Peters, *J. Chromatogr.*, **316**, 397 (1984).
[9]J. L. Meek, *Anal. Chem.*, **52**, 1370 (1980).
[10]H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, **485**, 27 (1989); see also optimization in Chapter 19.

10.3 SPECIAL SELECTIVITY EFFECTS FROM TERNARY SOLVENT MIXTURES[11]

If a relatively small amount (5–25%) of a third component is added to an optimum-strength methanol–water mixture, then special selectivity effects come into play, by means of which sample components with special functional groups are eluted at a specifically faster or slower rate:

(a) Acetonitrile as the third component accelerates compounds with the ester function —COOR selectively.
(b) Tetrahydrofurane accelerates compounds with methoxy groups $-OCH_3$ with respect to similar substances which do not contain such groups.
(c) Acetonitrile retains compounds containing methoxy groups.
(d) Dichloromethane accelerates compounds with chloro groups —Cl.
(e) Tetrahydrofuran accelerates straight-chain products with respect to cyclic compounds. Sorbic acid ($CH_3CH{=}CHCH{=}CHCOOH$) and benzoic acid (C_6H_5COOH) cannot be separated with a methanol–water mixture but a combination of 60% water (pH 2), 32% methanol and 8% tetrahydrofurane does achieve the desired effect.
(f) *N*,*N*-Dimethylformamide accelerates basic compounds (aromatic amines, *N*-heterocyclics) and reduces the amount of tailing.[12]

10.4 THE SELECTIVITY TRIANGLE IN REVERSED-PHASE CHROMATOGRAPHY

The selectivity concept, based on polarity, outlined in Section 9.4, is also applicable for reversed-phase chromatography. (Localization effects are unimportant.) Eluents representative of selectivity properties include methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF). As Fig. 10.4 shows, these three solvents are much more in the centre of the selectivity triangle than those for adsorption chromatography, i.e. they are less selective. The choice of solvent is severely restricted by the fact that it must be miscible with water.

Polarity can be adjusted by means of the weakest solvent, water. P' (reversed-phase) values are redefined, with the following values: water, 0; methanol, 2.6; acetonitrile, 3.2; and tetrahydrofuran, 4.5.

Problem 23

A sample mixture with reasonable k' values is eluted with 45% methanol in water, but the resolution is unsatisfactory. What other solvent mixtures should be tested?

[11]S. R. Bakalayar, R. McIllwrick and E. Roggendorf, *J. Chromatogr.*, **142**, 353 (1977).
[12]M. Ryba, *Chromatographia*, **15**, 227 (1982).

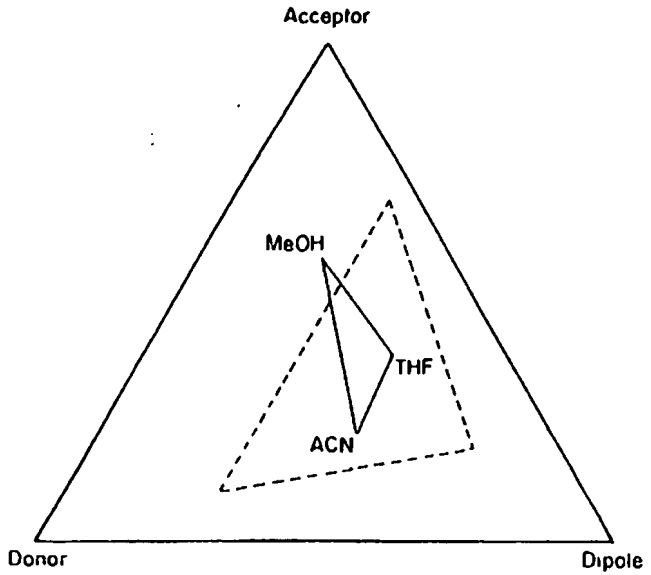

Fig. 10.4 Representatives of selectivity property (dashed line related to adsorption chromatography).

Solution

$$P'_{mixture} = P'_1\varphi_1 + P'_2\varphi_2$$

$$P'_{mixture} = P'_{MeOH} \times \varphi_{MeOH} (+ P'_{water} \times \varphi_{water}; \text{ this component is zero})$$
$$= 2.6 \times 0.45$$
$$= 1.17$$

Acetonitrile–water:

$$\varphi_{ACN} = \frac{P'_{mixture}}{P'_{ACN}} = \frac{1.17}{3.2} = 0.37$$
$$= 37\% \text{ ACN in water}$$

Tetrahydrofuran–water:

$$\varphi_{THF} = \frac{P'_{mixture}}{P'_{THF}} = \frac{1.17}{4.5} = 0.26$$
$$= 26\% \text{ THF in water}$$

10.5 STATIONARY PHASES[13]

Spatial dimensions dictate that not every silanol group in the silica will react and become converted into Si–O–Si–C during reversed-phase synthesis. However, the reaction must continue until the residual OH groups are fully screened by the C_{18} or C_8 tails; otherwise the product will retain its adsorptive properties (as described in Chapter 9). A stationary phase for reversed-phase chromatography should not adsorb methyl red, the test for which is very simple. The product is shaken in a solution of methyl red in toluene. Following a toluene wash, no trace of the reddish violet colour should remain.[14] A further test is to use hexane as the mobile phase and nitrobenzene as the sample, when the resulting k' value must be less than 0.5.[15]

As a general rule, retention times are longer the more C atoms the bonded stationary phase contains. (The reason is that the volume taken up by the bonded non-polar groups, i.e. that required by the actual stationary phase, is greater in long chains than it is in shorter chains; k' is directly proportional to the volume ratio between the stationary and mobile phases; see Section 2.3.) Figure 10.5 demonstrates this effect.

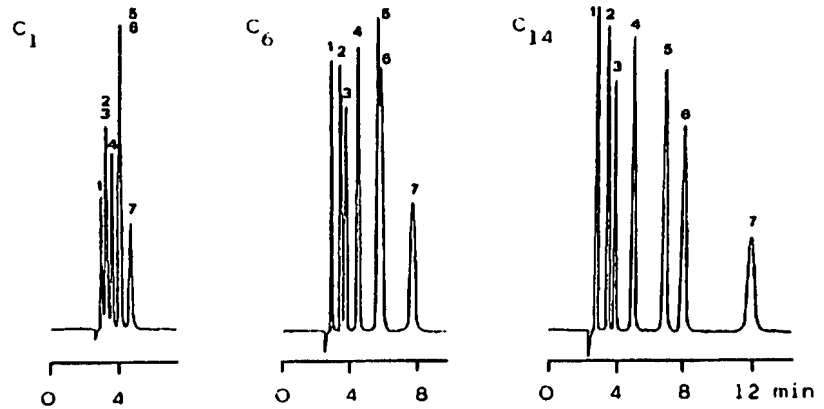

Fig. 10.5 Effect of chain length on retention (reproduced from G. E. Berendsen and L. de Galan, *J. Chromatogr.*, **196**, 21 (1980) by permission). Conditions: mobile phase, methanol–water (60:40). Peaks: 1 = acetone; 2 = *p*-methoxyphenol; 3 = phenol; 4 = *m*-cresol; 5 = 3,5-xylenol; 6 = anisole; 7 = *p*-phenylphenol.

[13]See also: K. K. Unger and K. D. Lork, *Eur. Chromatogr. News*, 2(2), 14 (1988); H. Engelhardt and M. Jungheim, *Chromatographia*, 29, 59 (1990).
[14]Quantitative methyl red test: I. Wouters, I. Quintens, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.*, 5, 25 (1982).
[15]Test for residual silanols with peptides: C. T. Mant and R. S. Hodges, *Chromatographia*, 24, 805 (1987).

Selectivity effects are also evident in stationary phases. Short alkyl chains are good for separating polar samples and long chains are better for non-polar substances (although non-polar components can be separated on C_8 columns of course and polar compounds on the C_{18} type also). Monomer layers are better for polar components and polymer layers suit non-polar components more (although polymer-bonded stationary phases generally have much poorer mass-transfer properties; on the other hand, their loadability is greater). Hence the potential of reversed-phase chromatography can only be fully exploited if a C_8 (or possibly a C_{18}) column packed with monomer stationary phase is used for separating polar compounds and a C_{18} column packed with polymer phase for non-polar samples.[16]

The type of stationary phase can be determined by using a test mixture of benzo[a]pyrene (BaP), 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN) and phenanthro[3,4-c]phenanthrene (PhPh) in acetonitrile.[17] The elution order of these three compounds gives information as follows:

BaP ≤ PhPh < TBN with $\alpha_{TBN/BaP} \geq 1.7$: monomeric C_{18} phase
PhPh < TBN ≤ BaP: polymeric C_{18} phase
PhPh < BaP < TBN with $\alpha_{TBN/BaP} < 1.7$: phase with intermediate properties such as a densely loaded monomeric or lightly loaded polymeric material

Many other test mixtures have been discussed. Engelhardt proposed the following one: toluene, ethylbenzene (hydrophobic interaction); phenol, ethylbenzoate (neutral, polar); aniline, *o*-, *m*-, *p*-toluidine, *N*,*N*-dimethylaniline (basic) and thiourea (inert).[18]

The phenyl phase has a different selectivity than the aliphatic hydrocarbon chains. The cyclohexyl phase is selective for all alicyclic compounds; the k' value of a cyclic compound is greater than that of its straight-chain isomers.

Stationary phases of the same type (e.g. C_{18}) offered by different manufacturers seem to be identical at first glance, but in fact they may have very different properties. The main reason for this is the variation in the starting materials (silicas with different specific surfaces) and the lack of standardization between chemical reaction methods. The most suitable phase may have to be established empirically in cases where separation is problematical. The stationary phase can undergo undesired interactions, especially with basic compounds which is observed as tailing. Strategies to solve such problems have been proposed.[19]

In contrast to adsorption chromatography, equilibrium between mobile and stationary phases is rapidly established in the reversed-phase method. When a solvent change is required, reproducible chromatographic results can be obtained after pumping through about 5-10 times the column volume of new eluent.[20]

Caution: C_{18} material decomposes at temperatures in excess of 200 °C!

[16] R. E. Majors, *J. Chromatogr. Sci.*, 15, 334 (1977).
[17] L. C. Sander and S. A. Wise, *J. of HRC*, 11, 383 (1988); Standard Reference Material 869 of the National Institute of Standards and Technology, Room B316, Chemistry Building, Gaithersburg, MD 20878, USA.
[18] H. Engelhardt, H. Löw and W. Götzinger, *J. Chromatogr.*, 544, 371 (1991).
[19] M. A. Stadalius, J. G. Berus and L. R. Snyder, *LC GC Int.*, 1(5), 22 (1988).

10.6 APPLICATIONS

Aqueous solutions such as those of biological origin, pharmaceutical formulations, drinks, etc., are common samples for analysis. As water is the weakest eluent, aqueous solutions may be injected directly without any special preliminary treatment (although filtration or centrifugation is strongly recommended). An example of the injection of 5 µl of drinks for caffeine determination was depicted in Fig. 1.2. Figure 10.6 provides a further example. Tranquillizer

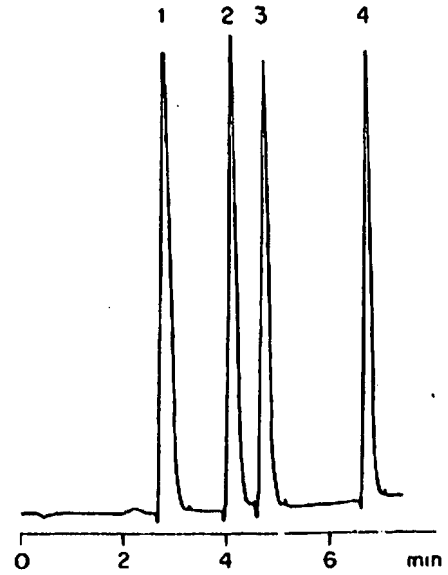

Fig. 10.6 Chromatogram of tranquillizers (Hewlett-Packard). Conditions: column, 25 cm × 4 mm i.d.; stationary phase, LiChrosorb RP-8, 10 µm; mobile phase, gradient elution, 30% acetonitrile in water up to 90% acetonitrile in water, 16 min; UV detector, 254 nm. Peaks: 1 = bromural; 2 = carbromal; 3 = acetocarbromal; 4 = benzyl mandelate.

[20] If all the eluents involved contain 3% of n-propanol the equilibration time is even shorter: L. A. Cole and J. G. Dorsey, *Anal. Chem.*, 62, 16 (1990).

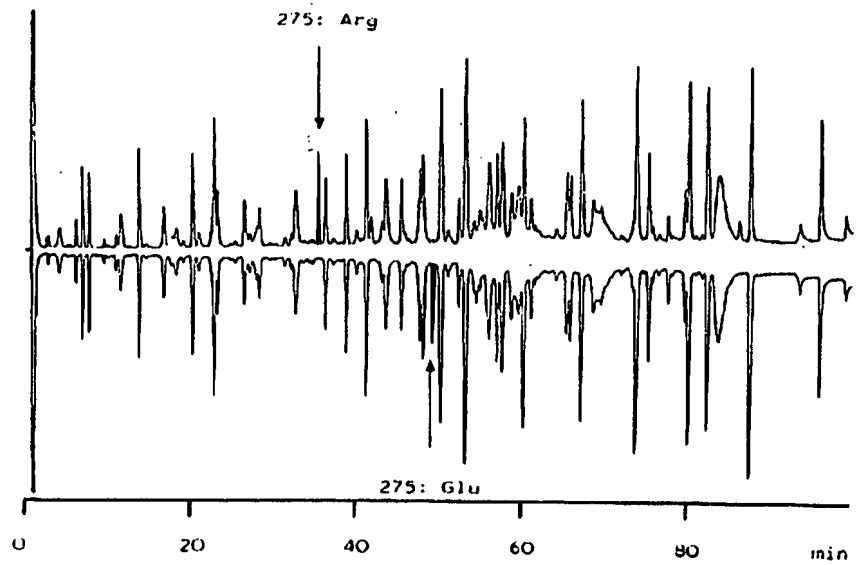

Fig. 10.7 Separation of the tryptic hydrolysate of tissue-type plasminogen activator (R. L. Garnick, N. J. Solli and P. A. Papa, *Anal. Chem.*, **60**, 2546 (1988)). Conditions: stationary phase, Nova Pak C18, 5 µm; mobile phase: 1 ml min^{-1} 50 mM sodium phosphate pH 2.8–acetonitrile, step gradient; UV detector 210 nm. Top: the normal protein with arginine at position 275; below: the mutant with glutamic acid at position 275.

tablets were dissolved in water, filtered and then injected. All other methods for the analysis of this type of drug take far longer.

Figure 10.7 shows the excellent performance of reversed-phase HPLC in biotechnological research. It represents the separation of the tryptic hydrolysate of the normal form and of a mutant of tissue-type plasminogen activator. This protein is built up of 527 amino acids and has a mass of approximately 67 000 dalton. The mutant differs in one single amino acid which leads to a deviating retention time of this specific fragment.

However, reversed-phase chromatography is not just suitable for polar compounds alone. For example, the seventeen polycyclic aromatic hydrocarbons which are referred to by environmental analysts as 'priority pollutants' can be completely separated within 15 min, as shown in Fig. 10.8. Figure 10.9 shows the separation of the complex mixture of aromatic gasoline components with a highly resolved elution pattern usually only expected from capillary gas chromatography.

Further examples of reversed-phase chromatography are given in Figs. 1.2, 2.15, 2.17, 5.12, 8.2, 15.4, 16.11b, 19.10, 19.11, 22.5, 23.2, 23.4, 23.8 and 23.10.

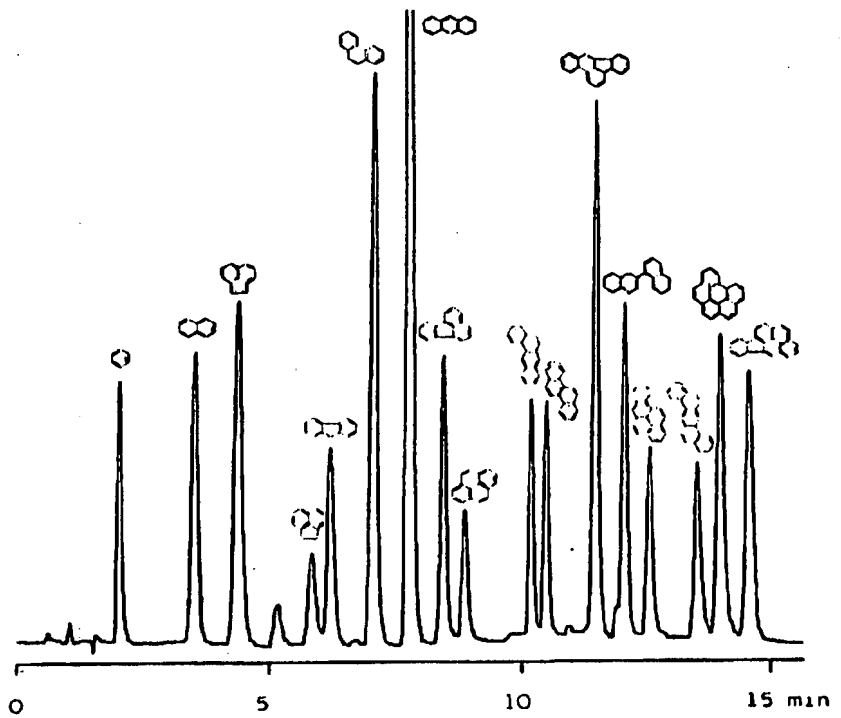

Fig. 10.8 Separation of polycyclic aromatic hydrocarbons (Separations Group). Conditions: column, 15 cm × 4.6 mm, i.d.; stationary phase, Vydac TP C_{18}, 5 µm; mobile phase, 1.5 ml min^{-1} water–acetonitrile, linear gradient from 50 to 100% acetonitrile between 3 and 10 min; UV detector, 254 nm.

10.7 HYDROPHOBIC INTERACTION CHROMATOGRAPHY[21]

The interaction between the sample and the stationary phase is so strong in reversed-phase chromatography that an aqueous eluent is too weak without the addition of an organic solvent. However, organic mobile phases are not allowed in certain cases of protein separation because of the risk of denaturation and subsequent loss of biological activity.

[21] B. F. Roettger and M. R. Ladisch, *Biotechnol. Adv.*, 7, 15 (1989); N. Cooke, P. Shieh and N. Miller, *LC GC Int.*, 3(1), 8 (1990) or *LC GC Mag.*, 7, 954 (1989).